

BRCA1 and BRCA2 bind Stat5a and suppress its transcriptional activity

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Abstract Germline mutations in the breast cancer susceptibility genes, *BRCA1* and *BRCA2*, are thought to account for a large portion of familial breast cancer. The increased risk of breast cancer in women carrying such mutations suggests that these proteins play a critical role in the growth regulation of mammary epithelial cells. Another protein, Stat5a, is known to be essential for growth and terminal differentiation of breast epithelial cells. Here we show that Stat5a forms a complex with both *BRCA1* and *BRCA2* in breast epithelial cells upon stimulation with prolactin. In addition, we show that the activity of Stat5a on the β -casein promoter is modulated by both *BRCA1* and *BRCA2*. This interaction may be important during the expansion and terminal differentiation of breast epithelial cells, as happens during pregnancy and lactation.

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1. Introduction

The breast cancer susceptibility genes *BRCA1* and *BRCA2* were identified by studies of families with inherited susceptibility to breast cancer. Mutations in these genes predispose to breast cancer as well as to other types of cancers such as prostate and ovarian cancer. Their normal protein products share many functional properties and both are involved in the fundamental cellular processes of maintaining genomic integrity, transcriptional regulation and embryonic cell proliferation (reviewed in [1]). It is not clear why mutations in these ubiquitously expressed genes, which participate in universal pathways, lead specifically to breast and ovarian cancer, but studies in mice have shown that expression of *BRCA1* and *BRCA2* is associated with the growth and differentiation of breast epithelial cells, especially during pregnancy and lactation. Both proteins are markedly up-regulated in the mammary gland early in pregnancy, during proliferation of breast epithelial cells as well as during differentiation to mature, milk-producing alveoli [2,3]. Increased knowledge about the normal functions of *BRCA1* and *BRCA2* proteins in signal transduction and growth regulation in breast cells may thus shed light on the pathogenesis of hereditary breast cancer and

reveal how direct or indirect inactivation of *BRCA* genes leads to breast tumorigenesis.

Stats (signal transducers and activators of transcription) are a family of transcription factors that mediate the transcriptional response to a diverse group of cytokines and growth factors. Upon binding these ligands, the receptors phosphorylate the Stats, which then dimerize, move to the nucleus and induce the transcription of specific genes [4]. Stat5a, also known as mammary gland factor, is activated by many cytokines and growth factors, including prolactin (PRL), growth hormone, and epidermal growth factor, and mediates the transcription of milk proteins such as β -casein, whey acidic protein and oncostatin M (reviewed in [4,5]). Studies of knockout mice have revealed that the *STAT5a* gene is necessary for normal development of the mammary gland. *STAT5a*^{−/−} mice are normal in size, weight and fertility, but female mice fail to lactate after parturition and are unable to nurse. The lobulo-alveolar units of the mammary tissue are extremely underdeveloped and do not show a secretory phenotype, even after maximal stimulation of PRL secretion [6]. Stat5a is strongly activated towards the end of pregnancy, persists in an activated state during pregnancy and lactation and is rapidly inactivated after cessation of suckling [7,8].

Stat5a, *BRCA1* and *BRCA2* are all up-regulated in the mammary gland during pregnancy and lactation and these proteins all have an important role in the normal development of breast epithelial cells. *BRCA1* and *BRCA2* are also known to interact with each other [9]. We therefore asked whether Stat5a interacted with *BRCA1* and *BRCA2* in breast epithelial cells.

2. Materials and methods

2.1. Cells and tissue culture

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. T47D and MCF-7 cells were cultured in RPMI medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and 10 μ g/ml insulin.

2.2. Antisera and reagents

The rat monoclonal antibody SC-3 was raised against the C-terminal amino acids 3386–3400 of human *BRCA2*. Rabbit antiserum against Stat5a was purchased from Santa Cruz and mouse monoclonal antibody against *BRCA1* (mAb1) was from Oncogene Research Products. Monoclonal anti-HA (3F10) was purchased from Boehringer Mannheim and monoclonal anti-flag (M5) was from Sigma. Peroxidase-conjugated pig anti-rabbit immunoglobulins and sheep anti-mouse immunoglobulin were from Amersham. The rabbit anti-

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serum PDGFR-33 has been described previously [10]. Ovine PRL (sheep luteotropic hormone) was purchased from Sigma.

2.3. Plasmids and cDNA transfections

The plasmids pMT-SMII and pMT-SMII/5'flag-BRCA2 have been previously described [11]. The plasmid pcDNA3β/5'HA-BRCA1 was provided by Ralph Scully, Dana Farber Institute, Boston, MA, USA. Stat5a cDNA (pXM-Stat5) [12] and β-casein promoter (pZZ1; -344 to -1 β-casein promoter 5' pLucDSS) [13] were provided by Bernd Groner, Institute for Biomedical Research, Georg-Spyer-Haus, Frankfurt, Germany. pcDNA3-PDGF β-receptor was provided by Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden. pCH110 encoding the β-galactosidase gene under the control of the SV40 promoter was from Pharmacia Biotech. Transient transfections were performed with Fugene-6 (Boehringer Mannheim) using 0.7 μg of each cDNA and keeping the total amount of cDNA constant using empty vector.

2.4. Cell lysis and immunoprecipitation

T47D cells and MCF-7 cells were starved for 24 h, and then stimulated with 5 μg/ml PRL (sheep luteotropic hormone) for different time periods at 37°C. After washing with phosphate-buffered saline (PBS), cells were solubilized in lysis buffer containing 1% Triton X-100, 1% sodium deoxycholate, 10% glycerol, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 100 μM NaVO₃. The lysates were centrifuged and the supernatants immunoprecipitated with specific antibodies for 2 h at 4°C, followed by incubation with protein G-Sepharose 4 Fast Flow (Amersham Pharmacia Biotech AB) for 30 min. Immunoprecipitates were washed twice with lysis buffer, eluted from the Sepharose beads and analyzed by SDS-gel electrophoresis in a gradient gel of 5–10% polyacrylamide. The samples were electrophoretically transferred to Immobilon-Blot polyvinylidene difluoride membrane (Bio-Rad) at 400 mA for 3–4 h at 4°C in 20% methanol, 0.2 M glycine, and 25 mM Tris-HCl. Blots were blocked by incubation in PBS containing 5% dried milk and 0.05% Tween 20 (Merck) for 1 h at room temperature before probing with specific antibodies. After incubation with different antisera, the blots were washed three times in PBS containing 0.05% Tween 20 and then incubated with the peroxidase-conjugated pig anti-rabbit or sheep anti-mouse immunoglobulins (1:5000 and 1:1000 dilutions, respectively). After washing, bound antibodies were visualized using the ECL Western blotting detection system.

2.5. Luciferase and β-galactosidase assays

COS-7 cells or T47D cells were left untreated or stimulated with either 50 ng/ml PDGF-BB or 5 μg/ml PRL for 8–16 h. Then β-galactosidase and luciferase activities were determined on triplicate samples using the β-galactosidase and luciferase assay systems (Promega) as described by the manufacturer. Luciferase activities were corrected for the β-galactosidase values obtained in each experiment.

3. Results

To test for potential interaction between BRCA1 and Stat5a, COS-7 cells were transfected with cDNAs encoding *STAT5a* and HA-BRCA1, either alone or in combination

with each other. Forty-eight hours after transfection, the cells were lysed to obtain cell extracts, which were incubated with anti-HA mAb. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with antiserum against Stat5a. As shown in Fig. 1A, Stat5a co-precipitated with

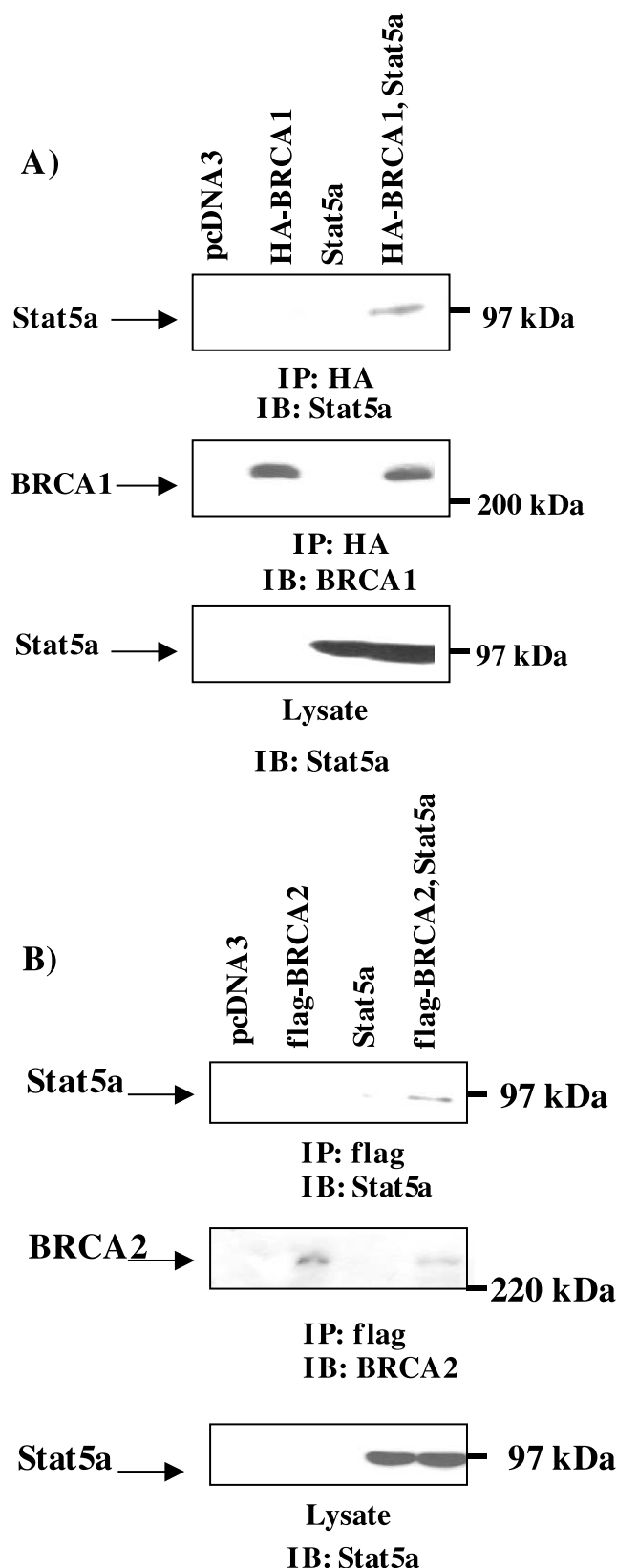


Fig. 1. Stat5a co-precipitates with BRCA1 and BRCA2 in transiently transfected COS-7 cells. A: COS-7 cells were transiently transfected with pcDNA3 alone, BRCA1-HA, Stat5a or a combination of BRCA1-HA and Stat5a. After 48 h, the cells were lysed and immunoprecipitated with anti-HA, followed by immunoblotting with anti-Stat5a (upper panel) and BRCA1 (middle panel). In parallel, expression levels of Stat5a were assessed by immunoblotting 10% of the cell lysate with anti-Stat5a (bottom panel). B: The experiments described in A were repeated using pcDNA3 alone, Flag-BRCA2, STAT5a or a combination of Flag-BRCA2 and Stat5a. The cell lysates were then immunoprecipitated (IP) with anti-Flag, followed by SDS-PAGE and immunoblotting (IB) with anti-Stat5a (upper panel) and anti-BRCA2 (middle panel). In addition, expression levels of Stat5a were assessed by immunoblotting 10% of the cell lysate with anti-Stat5a (bottom panel).

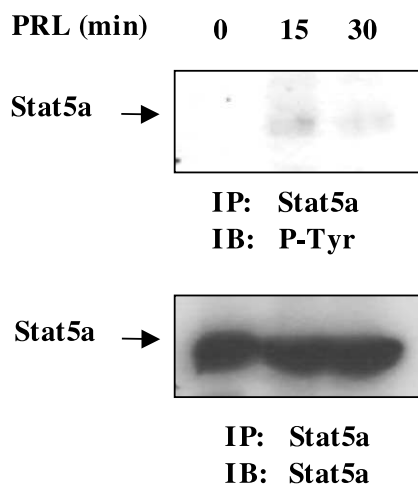


Fig. 2. Tyrosine phosphorylation of Stat5a in response to PRL. Serum-starved T47D cells were stimulated with 5 μ g/ml ovine PRL, for 15 or 30 min at 37°C. Cells were lysed and immunoprecipitated with anti-Stat5a, followed by SDS-PAGE and immunoblotting with anti-phosphotyrosine (upper panel), and anti-Stat5a (lower panel).

HA-BRCA1 in cells overexpressing these two proteins. In addition, 10% of the cell lysate was immunoblotted with anti-Stat5a to confirm equal expression of the transfected protein (Fig. 1A, bottom panel). To investigate whether Stat5a also co-precipitated with BRCA2, COS-7 cells were transfected with plasmids encoding *STAT5a* and flag-*BRCA2*, either alone or in combination with each other. As shown in Fig. 1B, Stat5a co-precipitated with flag-*BRCA2* in cells overexpressing these proteins. As before, 10% of the cell lysate was immunoblotted with anti-Stat5a (Fig. 1B, bottom panel) to confirm equal expression of the transfected protein.

We then asked if Stat5a could interact with BRCA1 and BRCA2 under endogenous conditions. Also, we wanted to examine if such an interaction was induced by tyrosine phosphorylation of Stat5a. For this purpose, we used the breast cancer cell lines MCF-7 and T47D, which have been shown to express PRL receptors on the cell surface [14] as well as endogenous Stat5a, BRCA1 and BRCA2 [15–17]. To examine whether tyrosine-phosphorylated Stat5a was detected in these cells upon short-term ligand stimulation, serum-starved T47D cells were stimulated with 5 μ g/ml ovine PRL for 15 and 30 min at 37°C. Stat5a was immunoprecipitated from the cell lysates with specific anti-Stat5a antiserum, followed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody. As shown in Fig. 2, tyrosine-phosphorylated Stat5a was detected upon stimulation with PRL for 15 min, in agreement with previous studies [15]. Similar results were obtained using MCF-7 cells (data not shown).

In order to estimate if Stat5a formed an endogenous complex with BRCA1, MCF-7 cells were serum-starved and then stimulated with 5 μ g/ml ovine PRL for 15 and 30 min at 37°C. BRCA1 was immunoprecipitated from the cell lysates with anti-BRCA1 mAb. Precipitated proteins were analyzed by SDS-PAGE, followed by immunoblotting with anti-BRCA1 (Fig. 3A, upper panel) or anti-Stat5a (Fig. 3A, middle panel), showing that a BRCA1–Stat5a complex was indeed present in the breast cells upon ligand stimulation. 10% of the cell lysates were run in parallel and immunoblotted with anti-Stat5a to confirm the expression of Stat5a in all lanes (Fig. 3A, bottom panel). To examine the specificity of the reaction, cell lysates

from PRL-stimulated T47D cells were immunoprecipitated with anti-BRCA1 mAb or anti-HA mAb, the latter was used as a negative control. The precipitated proteins were analyzed by SDS-PAGE, followed by immunoblotting with

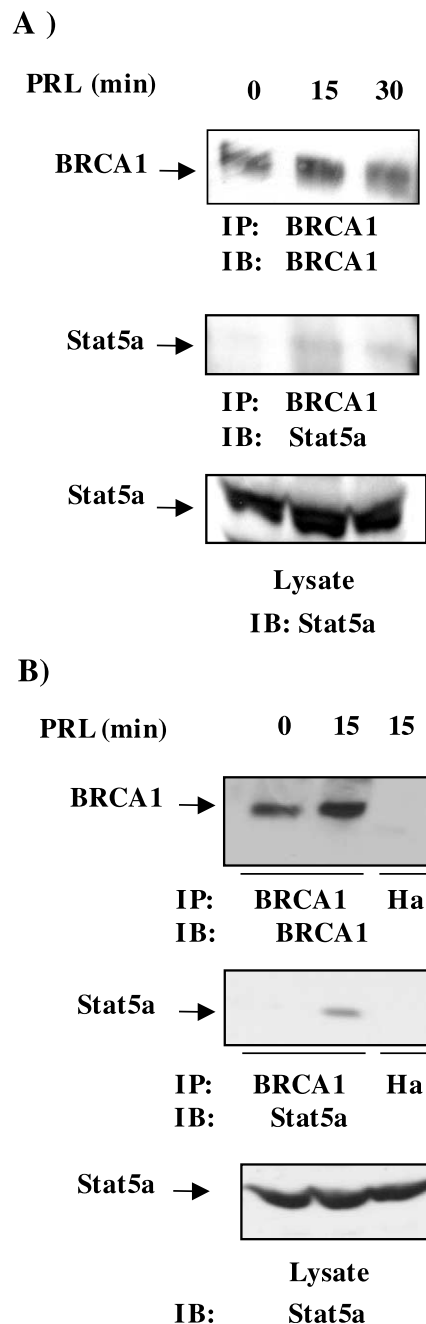


Fig. 3. Stat5a co-precipitates with BRCA1 in PRL-stimulated breast cells. A: Serum-starved MCF-7 cells were stimulated with 5 μ g/ml ovine PRL for 15 or 30 min at 37°C. Cells were lysed and immunoprecipitated (IP) with anti-BRCA1 mAb, followed by SDS-PAGE and immunoblotting (IB) with anti-BRCA1 (upper panel) or anti-Stat5a (middle panel). In parallel, expression levels of Stat5a were assessed by immunoblotting 10% of the cell lysate with anti-Stat5a (bottom panel). B: As in A, except that T47D cells were cultured in RPMI medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and 10 μ g/ml insulin. After serum starvation for 24 h, the cells were stimulated with 5 μ g/ml ovine PRL for 15 min and immunoprecipitated (IP) with anti-BRCA1 (lanes 1 and 2) or anti-HA (lane 3), followed by immunoblotting with anti-Stat5a.

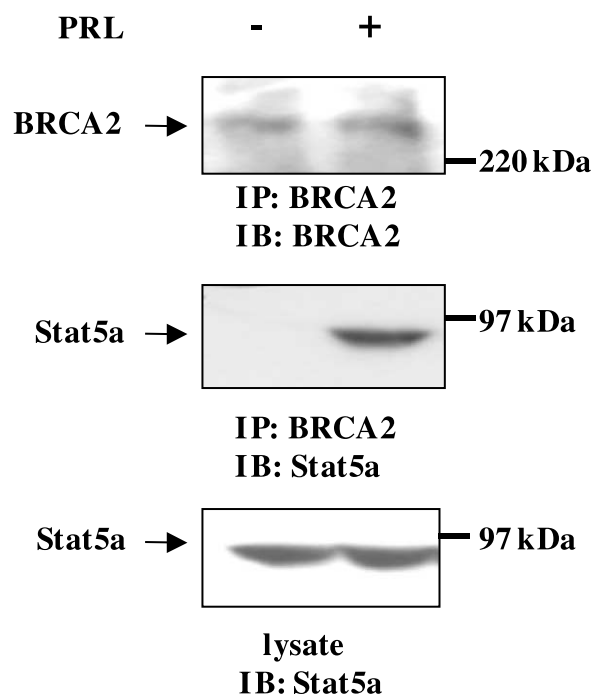


Fig. 4. Stat5a co-precipitates with BRCA2 in PRL-stimulated MCF-7 cells. Serum-starved MCF-7 cells were stimulated with 5 μ g/ml ovine PRL for 15 min at 37°C. Cell lysates were immunoprecipitated (IP) with anti-BRCA2 mAb (Sc-3), followed by SDS-PAGE and immunoblotting (IB) with anti-BRCA2 (Sc-3) (upper panel) or anti-Stat5a (middle panel). In parallel, 10% of the lysate was immunoblotted with anti-Stat5a (bottom panel) to confirm expression of the protein in all lanes.

anti-BRCA1 (Fig. 3B, upper panel) or anti-Stat5a (Fig. 3B, middle panel). Endogenous Stat5a co-precipitated specifically with anti-BRCA1 antibodies in response to PRL, whereas anti-HA mAb did not precipitate Stat5a. In addition, 10% of the protein lysate was immunoblotted with Stat5a, to check protein levels (Fig. 3B, bottom panel).

To determine whether endogenous Stat5a also forms a physical complex with BRCA2 in breast cancer cells, BRCA2 was immunoprecipitated with an anti-BRCA2 mAb (Sc-3) from extracts of MCF-7 cells. As shown in Fig. 4, middle panel, Stat5a indeed precipitated with BRCA2 after 15 min stimulation with PRL at 37°C. The filter was also immunoblotted with anti-BRCA2 (Fig. 4, top panel) and 10% of the protein lysate was immunoblotted with Stat5a, to check protein levels (Fig. 4, bottom panel). Both BRCA1 and BRCA2 thus appear to form a physical complex with Stat5a in breast epithelial cells upon ligand stimulation.

Stat5a dimers bind the β -casein promoter on ligand stimulation, increasing expression of the gene. To examine whether the interaction between BRCA2 and Stat5a affected the latter's transcriptional activity, COS-7 cells were transfected with the β -casein promoter driving a luciferase reporter (pZZ1), together with different combinations of cDNAs encoding *STAT5a*, *PDGF β -receptor* or *BRCA2*, using a plasmid encoding a β -galactosidase reporter (CH-110) as a control for transfection efficiency. The total amount of transfected DNA was kept constant using empty vector. As shown in Fig. 5A, stimulation with PDGF-BB induced transcriptional activity of exogenous Stat5a, in agreement with previous studies [18]. This induction was significantly decreased in cells overexpress-

ing BRCA2 protein (Fig. 5A, lane 6), although transfection with *BRCA2* alone had no effect on expression of the reporter (Fig. 5A, lane 4).

To investigate whether both BRCA1 and BRCA2 could modulate the transcriptional activity of Stat5a in breast cell lines, T47D cells were transiently transfected with the reporters used in the previous experiment together with different combinations of cDNAs encoding *STAT5a*, the *PDGF β -receptor*, *BRCA1* or *BRCA2*. As shown in Fig. 5B, the transcriptional activity of Stat5a was markedly decreased in cells overexpressing either *BRCA1* or *BRCA2*. To investigate whether this inhibition also affected the Stat5a activity induced by PRL treatment, T47D cells were transfected with the β -casein and control reporters together with different combinations of cDNAs encoding *STAT5a*, *BRCA1* or *BRCA2*. The cells were either left untreated or stimulated with PRL for 16 h. The PRL-induced transcription of the β -casein reporter was markedly decreased by co-transfection of either *BRCA1* or *BRCA2* (Fig. 5C). As shown in Figs. 1A and 1B (lanes 3 and 4), co-transfection of *BRCA1* or *BRCA2* had no effect on the level of ectopic Stat5a expressed in COS-7 cells, excluding the possibility that the inhibition of Stat5a transcriptional activity resulted from changes in the level of Stat5a. Neither BRCA1 nor BRCA2 proteins had any toxic effects when overexpressed as judged by flow cytometry (data not shown). The transcriptional activity of Stat5a induced by both PRL and PDGF thus appears to be inhibited by BRCA1 and BRCA2 in cells overexpressing these proteins.

4. Discussion

We have shown that the transcription factor Stat5a interacts with BRCA1 and BRCA2 after stimulation with PRL. Such stimulation induces tyrosine phosphorylation of Stat5a, which is followed by dimerization and translocation into the nucleus [15]. The interaction was also detected without ligand stimulation, in COS-7 cells overexpressing these proteins, however, the COS-7 cells were not serum-starved before lysis, giving the possibility that cytokines in the medium induced tyrosine phosphorylation of Stat5a.

The increased risk of breast cancer in women carrying germline mutations in *BRCA1* and *BRCA2* suggests that these proteins play a critical role in the growth regulation of mammary epithelial cells, perhaps regulating signaling pathways during pregnancy and lactation. The mammary gland develops at puberty and undergoes further changes during and after each pregnancy. The initial rapid proliferation of the breast epithelium is followed by differentiation during the later stages of pregnancy. The development of the mammary gland reflects the precise interplay of multiple hormones such as estrogens, growth hormones, insulin and corticosteroids which synergize with PRL in the development of the fully functional mammary gland. Lactation is initiated as a result of increasing PRL concentrations whereas glandular involution is induced by decreasing concentrations of PRL and postlactational apoptosis [8].

Most breast cancers originate in the terminal end ducts or intralobular terminal ducts. These structures are most numerous in nulliparous women and are lost on differentiation during pregnancy and lactation. An early age at first full-term pregnancy and increased parity are both associated with long-term reductions in the risk of breast cancer. After preg-

nancy, the risk of breast cancer transiently increases, but it subsequently remains lower for a period extending into the postmenopausal years [19,20]. In carriers of germline mutations in *BRCA1* and *BRCA2*, however, pregnancy may increase the risk of breast cancer, according to a study by Jernström et al. [20].

BRCA1 and *BRCA2* function as transcriptional co-regulators through direct interaction with sequence-specific transcription factors and with components of the transcriptional machinery. In addition, chromatin remodeling functions have been attributed to both *BRCA1* and *BRCA2* (reviewed in [1]). In our study, *BRCA1* and *BRCA2* proteins suppressed the transcriptional activity of Stat5a on the β -casein promoter. The mechanism by which *BRCA1* and *BRCA2* suppress Stat5a activity remains to be elucidated. They do not appear to decrease the level of the Stat5a protein (Fig. 1A,B, bottom panel) or its DNA binding activity (results not shown), and may instead modulate its capacity to affect transcription. Other transcription factors are known to influence the activity of Stat5. Thus, for example, the glucocorticoid receptor binds to Stat5 and enhances its transcriptional activity, while proteins such as PIAS and Socs inhibit its transcriptional activity through different mechanisms [5]. It is not known whether any of these proteins interact with *BRCA1* or *BRCA2*. *BRCA1* has previously been found to act in concert with Stat1 to activate transcription of interferon- γ target genes and mediate growth inhibition by this cytokine. In that study, *BRCA1* interacted directly with Stat1 [21]. Similarly, Stat1 was recently found to be up-regulated by *BRCA1*, in a *BRCA1*-inducible cell line [22]. Although Stat1 and Stat5 are structurally very similar, they seem to play different roles in cells. Thus, Stat1 has been suggested to induce growth inhibition and apoptosis in breast cells, whereas Stat5 promotes cellular proliferation and differentiation [23].

PRL, the main activator of Stat5a in mammary cells, has been shown to be one of the important growth factors that promote proliferation of breast cancer cells. PRL induces growth of mammary tumors in rodent models in vivo, and the PRL receptor has both autocrine and paracrine activity in breast cancer cells in vitro (reviewed in [24]). In addition, higher plasma levels of PRL are associated with an increased risk of breast cancer in postmenopausal women [25]. Recent studies have indicated that PRL activates the cyclin D1 promoter via the Jak-Stat pathway [26]. Cyclin D1, which is over-expressed in many breast tumors, has been suggested to play a

crucial role in the development of breast cancer malignancy [23]. Interestingly, cyclin D1 has recently also been shown to be transcriptionally down-regulated by *BRCA1* in *BRCA1*-inducible cells [22]. The results of our study indicate that *BRCA1* and *BRCA2* may help to limit the PRL-induced activation of Stat5a during periods such as glandular involution, when the activity of Stat5a is known to be down-regulated at

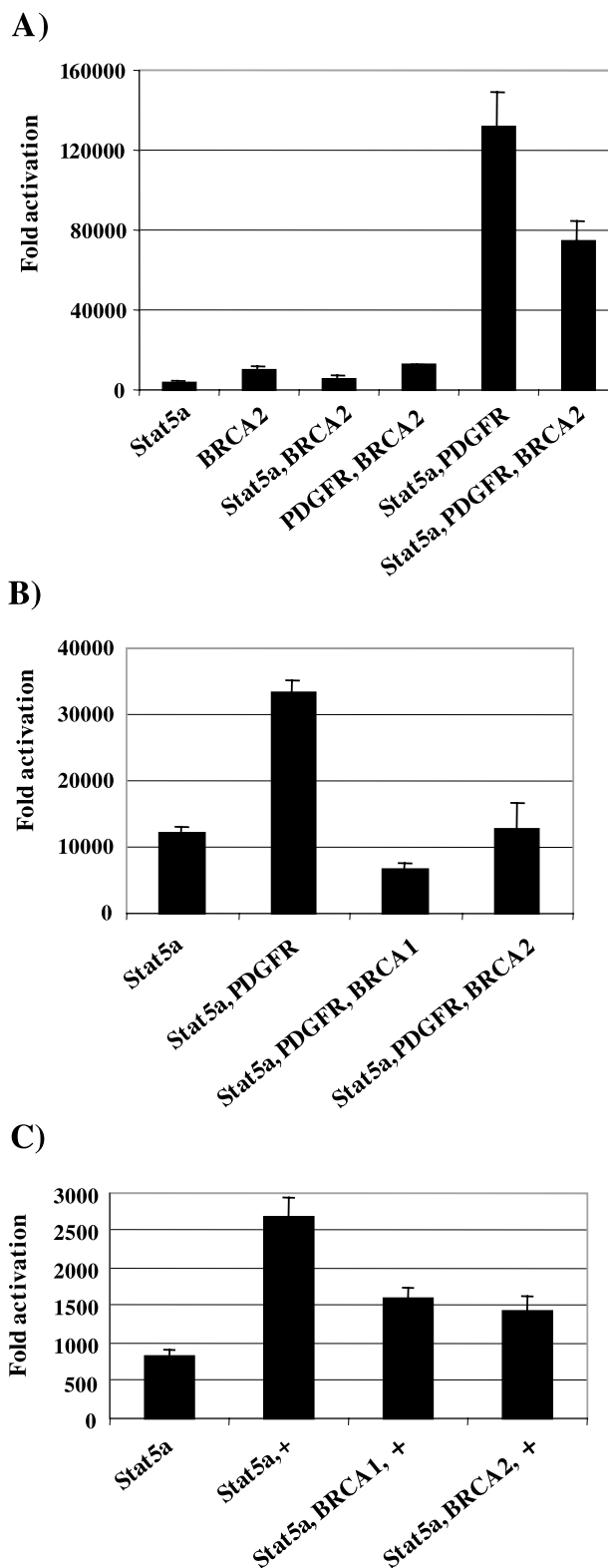


Fig. 5. Decreased transcriptional activity of Stat5a in cells overexpressing *BRCA1* or *BRCA2*. A: COS-7 cells transfected with β -casein promoter luciferase construct (all columns), β -galactosidase (pCH110, all columns), *STAT5a* cDNA (columns 1,3,5,6), *PDGFR* β -receptor (columns 4–6) or Flag-*BRCA2* (columns 2–4, 6) were stimulated with 100 ng/ml PDGF-BB for 8 h. The transcriptional activity of Stat5a on the β -casein promoter was determined by luciferase assay (PharMingen) in triplicate samples using β -galactosidase assays (PharMingen) as a control. B: T47D cells transfected with β -casein promoter luciferase construct, β -galactosidase (pCH110, all columns), *STAT5a* (all columns), *PDGFR* β -receptor (columns 2–4), HA-*BRCA1* (column 3), or Flag-*BRCA2* (column 4) were stimulated with 100 ng/ml PDGF-BB for 8 h. The transcriptional activity of Stat5a on the β -casein promoter was determined by luciferase assay as before. C: As in B, except that T47D cells were stimulated with 5 μ g/ml PRL (+, columns 2–4) for 16 h and transcriptional activity of Stat5a determined by luciferase assay and β -casein promoter.

the same time as the expression of BRCA1 and BRCA2 is increased [3].

In conclusion, we have shown that both BRCA1 and BRCA2 associate with Stat5a on ligand stimulation and modulate its transcriptional activity. This interaction may be important for the terminal differentiation of breast epithelial cells during pregnancy and lactation, and its failure may play a role in the pathogenesis of breast cancer.

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